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Arthropods Associate with their Red Wood ant Host without Matching Nestmate Recognition Cues

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Abstract Social insect colonies provide a valuable resource that attracts and offers shelter to a large community of arthropods. Previous research has suggested that many specialist parasites of social insects chemically mimic their host in order to evade aggression. In the present study, we carry out a systematic study to test how common such chemical deception is across a group of 22 arthropods that are associated with red wood ants (*Formica rufa* group). In contrast to the examples of chemical mimicry documented in some highly specialized parasites in previous studies, we find that most of the rather unspecialized red wood ant associates surveyed did not use mimicry of the cuticular hydrocarbon recognition cues to evade host detection. Instead, we found that myrmecophiles with lower cuticular hydrocarbon concentrations provoked less host aggression. Therefore, some myrmecophiles with low hydrocarbon concentrations appear to evade host detection via a strategy known as chemical insignificance. Others showed no chemical disguise at all and, instead, relied on behavioral adaptations such as particular defense or evasion tactics, in order to evade host aggression. Overall, this study indicates that unspecialized myrmecophiles do not require the matching of host recognition cues and advanced strategies of chemical mimicry, but can integrate in a hostile ant nest via either chemical insignificance or specific behavioral adaptations.

Keywords Ant guests · Aggression · Chemical mimicry · Inquilines · Myrmecophiles · Social parasitism

Introduction

Organisms throughout the animal and plant kingdom use a variety of chemical strategies to deceive other species. They produce signals that mask their true nature from the target species, thereby tricking them to believe they are mating partners, nestmates, harmless or even mutualistic (Wyatt 2012). Spectacular examples can be found in *Mastophora* bolas spiders that lure and prey on male moths by imitating the female moth sex pheromone (Eberhard 1977) or in the pitchers of carnivorous plants that release flowery odors to trap insects (Joel 1988). Chemical deception, however, has been most thoroughly explored in parasites of social insects (Lenoir et al. 2001; Akino 2008; van Zweden and D'Ettorre 2010). Previous studies have shown that many arthropods succeed in penetrating the heavily defended fortresses of various species of social insects and avoid being aggressed by matching the chemical profile of their social insect hosts (Nash and Boomsma 2008; van Zweden and d'Ettorre 2010). Such deception can occur by passively acquiring the host's cuticular hydrocarbons (CHCs) that are used in nestmate recognition ("chemical camouflage") or in some cases even by actively producing them ("chemical mimicry") (Nash and Boomsma 2008; van Zweden and d'Ettorre 2010). In the present paper, we will refer to both types of matching as chemical mimicry, because only few studies have conclusively demonstrated the mechanism by which the matching of the host profile is achieved (see Table 1). In some cases it has also been reported

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that the parasites release specific secretions that appease, repel or manipulate the host (Hölldobler and Wilson 1990; Thomas et al. 2002; Akino 2008). Another strategy used in some species is one of "chemical insignificance", whereby arthropods suppress the production of hydrocarbons used in nestmate recognition in order to escape detection (Lenoir et al. 2001; Witte et al. 2008; Lenoir et al. 2013) (Table 1). Lastly, in a strategy known as chemical transparency, it was suggested that some species can integrate in social insect colonies by suppressing the production of some specific key host recognition cues (Cervo et al. 2008; Martin et al. 2008a).

Animals living inside the nest of social insects are known as inquilines. The group of arthropod inquilines associated with social insects comprise parasitic social insects, here referred to as "social inquilines", and a wide variety of non-social arthropods (Kistner 1982; Hölldobler and Wilson 1990). Current data indicate that chemical mimicry is especially common among social inquilines that are phylogenetically related to their host (Nash and Boomsma 2008; Buschinger 2009) Surprisingly, complete chemical mimicry or mimicry of some specific key recognition cues has also been reported as an integration mechanism in most of the non-social inquiline arthropods that live inside social insect colonies and which are all very distantly related to their host. In fact, chemical mimicry has been claimed to occur in 50 out of 61 inquilines of which the CHC composition has been compared to that of their social insect host (reviewed in Table 1). Nevertheless, most of these studies are biased in the sense that they have focused mainly on highly specialized parasites. We define specialists as integrated species that closely interact with their host, have a narrow host distribution, may engage in regular grooming interactions with their host, solicit for food and are often treated as regular colony members (Hölldobler and Wilson 1990; Kronauer and Pierce 2011; Parker 2016). The majority of such species are obligate parasites that feed on brood, steal food or live as ectoparasites on the brood (Hölldobler and Wilson 1990). In many cases, these specialized arthropods also combine complex chemical adaptations with other specialized behavioral or morphological adaptations, e.g. in some cases allowing for acoustical mimicry (Hölldobler and Wilson 1990; Di Giulio et al. 2015; Parker 2016). The true incidence of chemical mimicry of species living in social insect nests, however, may well be lower than presumed as there is also a large group of unspecialized associates, whose cuticular hydrocarbon profiles have as yet hardly been investigated. Such unspecialized species are not accepted in the colony by being groomed, fed or transported in contrast to specialized symbionts (Kistner 1982; Hölldobler and Wilson 1990) and provoke aggression to a varying degree (Parmentier et al. 2016b). They typically have a broad host distribution and are very similar in morphology to free-living relatives (Parmentier et al. 2014) and tend to live as facultative parasites, scavenging in the nest (Parmentier et al. 2016a).

Red wood ants (RWAs) (*Formica rufa* group) support a very diverse community of obligately associated arthropods. These arthropods are typically unspecialized and feed on other myrmecophiles, prey remnants or brood (Parmentier et al. 2016a). Most RWA myrmecophiles live as inquilines inside the nest, whereas some live extranidally in the close vicinity of the nest (Parmentier et al. 2014). Besides obligate myrmecophiles, RWA nests also host a wide array of facultatively associated myrmecophiles. These species are not strictly associated with ants, but often occur inside RWA mounds (Parmentier et al. 2014). A large group of myrmecophiles associated with RWAs can also be found in nests of other ant species (Parmentier et al. 2014).

The aim of the present study was to carry out a systematic study of the chemical integration mechanisms or strategies that weakly specialized RWA-associated arthropods use to evade host aggression. In order to do so, we analyzed the CHC profiles of the ant associates and compared their similarity to those of their host workers as well as the total amount of CHCs produced per surface area of the cuticle. Our hypothesis was that chemical mimicry would manifest itself in a high similarity in cuticular profiles between the ant associates and their host, whereas a quantitatively and qualitatively low similarity might point towards chemical insignificance. Both strategies are expected to result in lower host aggression. CHC similarity and concentration were, therefore, linked with host aggression for the myrmecophile community. The effect of CHC similarity and concentration was controlled with other functional traits, including host specificity, brood predation tendency, level of nest integration, trophic role and body size, investigated in this or previous studies.

Methods and Materials

Study System

As a study system we used red wood ants (*Formica rufa* group) and their community of associated arthropods. In our study region (Northern Belgium and Northern France), three of the six species of the *F. rufa* group can be found: *Formica rufa* Linnaeus, 1761, *Formica polyctena* Förster,1850 and *Formica pratensis* Retzius, 1783 (Dekoninck et al. 2010). They tend to differ in ecological preferences and in colonial organization (Seifert 2007), but all sampled colonies occurred along forest edges, contained multiple queens, and had polydomous colonies that extended over multiple mounds in our study region.

Table 1 Chemical integration strategies of non-social termite, ant, wasp and bee intranidal associates (= inquiline) described in the literature

Species *	Family	Strategy	Behavior	Hosts	References
TERMITOPHILE					
Coleoptera					
Philotermes howardi [a]	Staphylinidae	mimicry (S)	trophallaxis, grooming	*Reticulitermes virginicus*	Howard 1978; Howard et al. 1982
Trichopsenius depressus [a]	Staphylinidae	mimicry (S)	trophallaxis, grooming	*Reticulitermes virginicus*	Howard and Kistner 1978; Howard et al. 1982
Trichopsenius frosti [a]	Staphylinidae	mimicry (S)	trophallaxis, grooming, phoresy	*Reticulitermes flavipes*	Howard 1976; Howard et al. 1980
Xenistusa hexagonalis [a]	Staphylinidae	mimicry (S)	trophallaxis, grooming	*Reticulitermes virginicus*	Howard 1978; Howard et al. 1982
MYRMECOPHILE					
Acari					
Unknown [a]	Acari	insignificance (S)	phoretic	*Leptogenys distinguenda*	Witte et al. 2008
Araneae					
Cosmophasis bitaeniata [b]	Salticidae	mimicry (S)	feeds on ant larvae	*Oecophylla smaragdina*	Allan and Elgar 2001; Elgar and Allan 2004, 2006
Sicariomorpha maschwitzi [a]	Oonopidae	mimicry (part of explanation) (O)	crawling on workers, trail following	*Leptogenys distinguenda*	von Beeren et al. 2012a; Witte et al. 2009
Blattodea					
Attaphila sp. [a]	Ectobiidae	mimicry (B)	follow trails, cling to queens	*Acromyrmex octospinosus*	Nehring et al. 2016
Attaphila sp. [a]	Ectobiidae	mimicry (B)	follow trails, cling to queens	*Atta colombica*	Nehring et al. 2016
Coleoptera					
Thermophilum sexmaculatum [b]	Carabidae	mimicry (P)	the larva feeds on ant larvae	*Lasius niger*	Dinter et al. 2002
Diomus thoracicus [a]	Coccinellidae	mimicry (O)	the larva feeds on ant brood	*Wasmannia auropunctata*	Vantaux et al. 2010
Thorictus buigasi [a]	Dermestidae	mimicry + insignificance (O)	phoretic, cling to ant antennae	*Cataglyphis viatica*	Lenoir et al. 2013
Thorictus martinezi [a]	Dermestidae	mimicry (O)	phoretic, cling to ant antennae	*Caraglyphis lenoiri*	Lenoir et al. 2013
Thorictus sulcicollis [a]	Dermestidae	mimicry (O)	phoretic, cling to ant antennae	*Cataglyphis hispanica*	Lenoir et al. 2013
Sternocoelis hispanus [a]	Histeridae	mimicry (T)	licked by ants, climb on larvae, phoretic	*Aphaenogaster senilis*	Lenoir et al. 2012
Unknown	Ptiliidae	mimicry (S)	phoretic on larvae	*Leptogenys distinguenda*	Witte et al. 2008
Aenictobia fergusoni [b]	Staphylinidae	mimicry (S)	follow ant column, ignored by ants	*Aenictus* sp. 18a of SKY	Maruyama et al. 2009
Aenictobia thoi [b]	Staphylinidae	mimicry (S)	follow ant column, ignored by ants	*Aenictus laeviceps*	Maruyama et al. 2009
Aenictoteras malayensis [a]	Staphylinidae	mimicry (S)	follow ant column, palpated, myrmecomorph	*Aenictus gracilis*	Maruyama et al. 2009
Aenictoxenus sp. undescribed [a]	Staphylinidae	mimicry (S)	phoretic on ant's abdomen	*Aenictus* sp. 18a of SKY	Maruyama et al. 2009
Chitosa nigrita [b]	Staphylinidae	mimicry (T)	few interactions	*Aphaenogaster senilis*	Lenoir et al. 2012
Diaritiger fossulatus [a]	Staphylinidae	mimicry (T)	food begging	*Lasius fuliginosus*	Akino 2002
Mimaenictus wilsoni [a]	Staphylinidae	mimicry (S)	follow ant column, transported, palpated, myrmecomorph	*Aenictus laeviceps*	Maruyama et al. 2009
Myrmecaphodius excavaticollis [a]	Scarabaeidae	mimicry (P)	grooming, trophallaxis	*Solenopsis*	Vander Meer and Wojcik 1982
Rosciszewskia gracilis [a]	Staphylinidae	mimicry (S)	trail following, myrmecomorph	*Aenictus gracilis*	Maruyama et al. 2009
Trachydonia marginalis [b]	Staphylinidae	mimicry (S)	trail following	*Leptogenys distinguenda*	Witte et al. 2008
Trichotobia gracilis [a]	Staphylinidae	2 peaks similar to larvae (S)	transported	*Aenictus gracilis*	Maruyama et al. 2009

Table 1 (continued)

Species *	Family	Strategy	Behavior	Hosts	References
Weissflogia rhopalogaster [a]	Staphylinidae	mimicry (S)	myrmecomorph, transported	*Aenictus* sp. 18a of SKY	Maruyama et al. 2009
Zyras comes [a]	Staphylinidae	mimicry (T)	food begging, trail following	*Lasius fuliginosus*	Akino 2002
Diptera					
Dohrniphora kistneri [b]	Phoridae	some similarity with ant larva (S)	follow ant column, not palpated	*Aenictus laeviceps*	Maruyama et al. 2009
Dohrniphora sp. 1 [b]	Phoridae	no mimicry (S)	follow ant column, not palpated	*Aenictus gracilis*	Maruyama et al. 2009
Dohrniphora sp. 2 undescribed [b]	Phoridae	mimicry (S)	follow ant column, not palpated	*Aenictus* sp. 18a of SKY	Maruyama et al. 2009
Puliciphora rosei [b]	Phoridae	insignificance (S)	trail following	*Leptogenys distinguenda*	Witte et al. 2008
Rhynchomicropteron necaphidiforme [b]	Phoridae	insignificance (S)	trail following	*Leptogenys distinguenda*	Witte et al. 2008
Vestigipoda maschwitzi [a]	Phoridae	some similarity with larvae (S)	mimicking of morphology of larva	*Aenictus gracilis*	Maruyama et al. 2009
Microdon albicomatus [a]	Syrphidae	mimicry (S)	specialized morphology, brood predator	*Myrmica incompleta*	Howard et al. 1990b
Microdon piperi [a]	Syrphidae	mimicry (S)	specialized morphology, brood predator	*Camponotus modoc*	Howard et al. 1990a
Microdon myrmicae [a]	Syrphidae	insignificance (S)	specialized morphology, brood predator	*Myrmica*	Witek et al. 2013
Gastropoda					
Allopeas myrmekophilos [a]	Subulinidae	insignificance (S)	transported	*Leptogenys distinguenda*	Witte et al. 2008
Hemiptera					
Paracletus cimiciformis [a]	Aphididae	mimicry (S)	specialized morph transported to brood	*Tetramorium*	Salazar et al. 2015
Hymenoptera					
Orasema sp. [a]	Eucharitidae	mimicry (P)	ectoparasite on ant larvae	*Solenopsis invicta*	Vander Meer et al. 1989
Dilocantha lachaudii [a]	Eucharitidae	partial mimicry (O)	adult transported outside nest	*Ectatomma tuberculatum*	Pérez-Lachaud et al. 2015
Isomerala coronate [a]	Eucharitidae	partial mimicry (O)	adult transported outside nest	*Ectatomma tuberculatum*	Pérez-Lachaud et al. 2015
Kapala sulcifacies [a]	Eucharitidae	mimicry (B/P/T)	larva attaches to workers, parasitizes larva	*Ectatomma ruidum*	Howard et al. 2001
Palaripsis eikoae [a]	Aphidiidae	mimicry (B/P)	mounting and rubbing against host, trophallaxis	*Lasius sakagamii*	Akino and Yamaoka 1998
Isopoda					
Exalloniscus maschwitzi [a]	Oniscidae	insignificance (S)	phoretic on pupae	*Leptogenys distinguenda*	Witte et al. 2008
Lepidoptera					
Maculinea alcon [a]	Lycaenidae	mimicry (S)	larva transported, tended and fed	*Myrmica rubra*, *M. scabrinodis*	Nash et al. 2008; Witek et al. 2013
Maculinea teleius [a]	Lycaenidae	partial mimicry (O)		*Myrmica*	Witek et al. 2013
Maculinea rebeli [a]	Lycaenidae	mimicry (T/P)	larva transported, tended and fed	*Myrmica schencki*	Akino and Knapp 1999; Schlick-Steiner et al. 2004; Schönrogge et al. 2004
Maculinea nausithous [a]	Lycaenidae	partial mimicry (O)		*Myrmica rubra*	Witek et al. 2013
Niphanda fusca [a]	Lycaenidae	mimicry (P)	carried to nest, trophallaxis	*Camponotus japonicus*	Hojo et al. 2009
Orthoptera					
Myrmecophilus sp. [a]	Myrmecophilidae	mimicry (P)	grooming, trophallaxis	several ants	Akino et al. 1996
Thysanura					
Malayatelura ponerophila [a]	Ateluridae	mimicry (P)	host rubbing	*Leptogenys distinguenda*	von Beeren et al. 2011; Witte et al. 2009

Table 1 (continued)

Species *	Family	Strategy	Behavior	Hosts	References
Unknown [b]		insignificance (S)	few interactions	*Aphaenogaster senilis*	Lenoir et al. 2012
Thysanura gen. sp. [a]		insignificance (S)	follow ant column, phoretic	*Aenictus* sp. 18a of SKY	Maruyama et al. 2009
BEE ASSOCIATE					
Acari					
Varroa jacobsoni [a]	Varroidae	mimicry (S)	specialized ectoparasite	*Apis mellifera*	Martin et al. 2001
Varroa destructor [a]	Varroidae	mimicry (P)	specialized ectoparasite	*Apis mellifera & Apis cerana*	Kather et al. 2015; Le Conte et al. 2015
Diptera					
Braula coeca [a]	Braulidae	mimicry of alkene part (T)	specialized parasite	*Apis mellifera*	Martin and Bayfield 2014
Hymenoptera					
Mutilla europaea [a]	Mutillidae	pre-integration: insignificance (S) post-integration: mimicry (P)	parasite enter wasp nests, lay eggs on host pupae and leave the nests	*Polistes biglumis*	Uboni et al. 2012; Uboni and Lorenzi 2013
Lepidoptera					
Acherontia atropos [b]	Sphingidae	partial mimicry (S)	facultative cleptoparasite of nectar and honey	*Apis mellifera*	Moritz 1991
WASP ASSOCIATES					
Coleoptera					
Metoecus paradoxus [a]	Rhipiphoridae	mimicry (S)	larva attaches to a foraging worker, parasitizes on wasp larva in nest	*Vespula vulgaris*	Van Oystaeyen et al. 2015

(*) Species that show integrated behavior, such as grooming, food soliciting, phoresy, ectoparasitism or myrmecomorphy were considered as specialists associates. These behavioural or morphological adaptations are absent, in the non-specialized red wood ant myrmecophile community, except for food begging in *Dinarda maerkelii*. Specialist species are indicated with [a] non-specialists with [b]. Different methods were used by the authors to support the claimed chemical strategy: (T) Statistical tests indicate that CHC profile of associate was more similar to host workers than to conspecific or congeneric workers. (B) Behavioural assay indicate that aggression of host colony workers was lower than aggression of alien colony workers. (P) CHC profile is more similar to the host in its presence. (O) The complete or partial CHC profile shows considerable overlap with the host, but the CHC profile can statistically be discriminated from the host colony. (S) Subjective judgement that the associate chemically mimicked the host or employs a strategy of chemical insignificance

Sample Collection

Myrmecophiles were collected from three different *F. rufa* populations: R1 (Boeschepe, 50°47′48.48″N, 2°40′31.00″E), R2 (Vladslo, 51°4′17.00″N, 2°55′44.27″E), R3 (West-Vleteren, 50°53′7.78″N, 2°41′50.92″E), six *F. polyctena* populations: O1 (De Haan, 51°16′4.72″N, 3°1′18.33″E), O2 (Beisbroek, 51°10′29.42″N, 3° 8′32.14″E), O3 (Beernem, 51°7′29.70″N, 3°20′4.37″E), O4 (Aartrijke, 51°8′39.56″N, 3° 4′58.91″E), O5 (Roksem, 51°10′27.26″N, 3° 3′6.60″E), and O6 (Herentals, 51°11′6.29″N, 4°48′34.99″E) and one *F. pratensis* population: P1 (Veltem-Beisem, 50°53′38.78″N, 4°38′6.74″E). In every population, we collected samples from a single polydomous colony. Nest material was taken at different locations in the nest and was gently spread out in a white plastic tray in the field. All myrmecophiles and ants were then collected by using an aspirator, of which the glass reservoir was regularly cleaned with hexane to minimize cross-contamination. An overview of the 18 collected inquiline or intranidal myrmecophiles with some life history traits are given in Table 2. In addition to these 18 RWA inquilines, three RWA myrmecophiles that live extranidally were collected: adults and larvae of the ladybird *Coccinella magnifica,* (Coccinellidae) (Sloggett et al. 1998) and adults of the leaf beetle *Clytra quadripunctata* (Chrysomelidae) (the larvae live intranidally) were captured on plants around RWA mounds, whereas the rove beetle *Pella humeralis* (Staphylinidae) that mostly scavenges in the neighbourhood of ant trails (Donisthorpe 1927), was found at the periphery of a RWA nest. Finally, two facultative myrmecophiles were collected: *Porcellio scaber* (the common rough woodlouse) and *Xantholinus linearis*, a rove beetle. In contrast to the myrmecophiles mentioned before, these species are typically not found in close contact with ants, but can occasionally also be found inside RWA mounds (Parmentier et al. 2014). Finally, we collected individuals of the ladybird *Coccinella septempunctata,* the free-living close relative of *C. magnifica*. Myrmecophiles and ants were kept together with some nest material and transferred with clean forceps to 2 ml glass vials (Sigma-Aldrich) in the lab for later chemical analysis. Animals were stored in the freezer at −18 °C until solvent extraction.

Chemical Analyses

CHCs from small myrmecophiles were extracted for 10 min in 30 μL of hexane (HPLC grade, Sigma-Aldrich) in 2 ml vials capped with a PTFE septum (Sigma-Aldrich). Large myrmecophiles, such as adults of *C. quadripunctata*, larvae of *C. quadripunctata*, adults of *C. magnifica*, larvae of *C. magnifica*, *C. septempunctata*, the isopod *P. scaber* and ant workers were extracted in 200 μL of hexane for 10 min. Samples were evaporated to dryness at room temperature in a laminar flow hood and stored at −18 °*C. prior* to analysis, samples were redissolved in 6 μL, 30 μL or 200 μL hexane. These different volumes were based on preliminary runs in which large variation in the total amounts of extracted CHCs were detected across our different samples. For small myrmecophiles or species with low hydrocarbon concentrations, CHCs of pools of 2 to 20 individuals were extracted per sample. 2 μL of each hexane extract was injected into a SHIMADZU QP 2010 ULTRA coupled gas chromatograph/mass spectrometer (GC/MS-system) equipped with a DB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm). Gas chromatographic conditions were: 1 min at 70 °C, two temperature ramps from 70 °C to 150 °C at 20 °C/min and from 150 °C to 320 °C at 3 °C/min, and the final temperature of 320 °C was held for 15 min. We used helium as the carrier gas at a flow rate of 1 mL/min, splitless injection, an inlet temperature of 280 °C, and a final pressure of 75 kPa. Mass spectrometry was performed with electron impact [EI] at 70 eV. In each batch we ran a linear C7 to C40 alkane ladder standard (49452-U, Supelco) at two different concentrations (0.001 μg/mL and 0.01 μg/mL). Since we earlier verified that the relationship between peak area and concentration was linear on a log-log scale, quantification of all hydrocarbons (total amount produced in ng per individual) was performed using interpolation on a log-log scale, using the peak areas of the closest eluting *n*-alkane of our external alkane ladders for each compound. Retention indices of all compounds were calculated using cubic spline interpolation (Messadi et al. 1990) based on the elution times of the external alkane ladders.

Peaks of CHCs were identified on the basis of expected mass spectrometric fragmentation patterns and retention indices provided by the NIST 2014 retention index database (available online in the NIST Chemistry Webbook, Linstrom and Mallard 2016) or by Pherobase (El-Sayed 2016). Retention indices and diagnostic ions are reported in Supplementary Material 1. Structure assignment of *n*-alkanes and monomethyl alkanes is straightforward and unambiguous (Carlson et al. 1998; Gerhardt et al. 2016). However, structure assignment of dimethylalkanes is more difficult as two different dimethyl alkanes can produce the same mass spectra (Gerhardt et al. 2016). In accordance with Gerhardt et al. (2016), we only considered dimethyl alkanes with an odd number of methylene groups between the branches, because they are biosynthetically most likely (Carlson et al. 1998). Double bond positions of alkenes of RWA workers were determined by using dimethyldisulfide (DMDS) derivatization of a pooled sample of 10 workers (Carlson et al. 1989). Diagnostic ions of DMDS alkene derivatives are given in Supplementary Material 1,. Unfortunately, we had too little material available to identify alkene double bonds of myrmecophiles using DMDS derivatization. Peak assignments were restricted to CHCs with chain lengths between *n*-C20 and *n*-C40 and which comprised more than 0.1% of the estimated

Table 2 Biology and functional traits of arthropods associated with red wood ants

Species	Taxon	Biology	Degree of host specificity	BC-dissimilarity to host workers		CHC concentration (ng/mm^2)		Brood predation tendency		Prop. in brood chamber		Trophic role	Prop. aggressive interactions	
				Mean [CI]	*N*	Mean [CI]	*N*	Mean [CI]	*N*	Mean [CI]	*N*		Mean [CI]	*N*
Red wood ant worker				0.07 [0.07-0.09]	46	228.6 [182.1-280.4]	36							
Amidobia talpa	Coleoptera (Staphylinidae)	O / I	3	0.63 [0.51-0.69]	4	36.1 [11.7-69.4]	4	0.18 [0.06–0.36]	22	0.11 [0.06–0.19]	106	S	0.12 [0.08–0.17]	22
Clytra quadripunctata (adult)	Coleoptera (Chrysomelidae)	O / E	3	0.44 [0.42-0.47]	5	289.0 [264.2-316.4]	5							
Clytra quadripunctata (larva)	Coleoptera (Chrysomelidae)	O / I	3	0.58	2	0.6 [0.5-0.6]	2	0.67 [0.48–0.83]	24	0.45 [0.30–0.61]	44	S	0.01 [0.00–0.03] *	10
Coccinella magnifica (adult)	Coleoptera (Coccinellidae)	O / E	4	0.55 [0.50-0.61]	13	204.2 [169.1-247.4]	13							
Coccinella magnifica (larva)	Coleoptera (Coccinellidae)	O / E	4	0.44 [0.43-0.45]	8	74.4 [42.6-117.4]	8							
Cyphoderus albinus	Collembola (Cyphoderidae)	O / I	1	0.39 [0.28-0.48]	3	26.2 [13.0-34.2]	3	0.00 [0.00-NA]	15	0.13 [0.06–0.23]	70	S	0.00 [0.00–0.02]	15
Dendrophilus pygmaeus	Coleoptera (Histeridae)	O / I	3	0.19 [0.18-0.20]	2	91.8 [65.5-118.2]	2	1.00 [NA-1.00]	9	0.00 [0.00–0.13]	26	S	0.19 [0.10–0.31]	6
Dinarda maerkelii	Coleoptera (Staphylinidae)	O / I	3	0.69 [0.65-0.75]	6	78.0 [50.6-101.3]	5	0.52 [0.33–0.72]	21	0.16 [0.07–0.30]	44	S	0.27 [0.21–0.33]	22
Emphylus glaber	Coleoptera (Cryptophagidae)	O / I	3	0.72 [0.72-0.72]	3								0.30 [0.16–0.50]	3
Leptacinus formicetorum	Coleoptera (Staphylinidae)	O / I	3	0.68 [0.59-0.68]	2	7.3 [7.1.-7.3]	2	0.81 [0.59–0.95]	16	0.12 [0.04–0.23]	52	H	0.42 [0.32–0.51]	11
Lyprocorrhe anceps	Coleoptera (Staphylinidae)	O / I	3	0.68 [0.61-0.74]	4	34.9 [10.6-44.6]	4	0.51 [0.36–0.67]	35	0.28 [0.16–0.42]	54	S	0.25 [0.19–0.31]	21
Mastigusa arietina	Araneae (Dictynidae)	O / I	2	0.72 [0.72-0.73]	5	1.3 [0.7-2.1]	5	0.10 [0.01–0.36]	10	0.00	15	H	0.73 [0.64–0.81]	12
Monotoma angusticollis	Coleoptera (Monotomidae)	O / I	4	0.72 [0.67-0.75]	8	1.5 [1.1-2.3]	8	0.68 [0.49–0.83]	25	0.23 [0.12–0.38]	47	S	0.03 [0.01–0.06]	20
Monotoma conicicollis	Coleoptera (Monotomidae)	O / I	4	0.48 [0.32-0.64]	2	4.1 [1.4-4.1]	2	0.50 [0.29–0.71]	18	0.33 [0.21–0.47]	55	S	0.05 [0.02–0.08]	20
Myrmetes paykulli	Coleoptera (Histeridae)	O / I	3	0.18 [0.14-0.21]	9	107.6 [79.3-134.9]	9	0.67 [0.46–0.83]	21	0.11 [0.04–0.25]	44	S	0.23 [0.13–0.25]	18
Notothecta flavipes	Coleoptera (Staphylinidae)	O / I	3	0.79 [0.77-0.80]	7	144.8 [103.0-188.4]	7	0.96 [0.83–1.00]	23	0.28 [0.15–0.44]	43	S	0.63 [0.56–0.70]	21
Pella humeralis	Coleoptera (Staphylinidae)	O / E	2	0.28	1	115.4	1						0.13 [0.06–0.24]	6
Platyarthrus hoffmannseggii	Isopoda (Platyarthridae)	O / I	1	0.61 [0.44-0.72]	8	7.7 [2.4-20.9]	7	0.60 [0.39–0.79]	20	0.25 [0.15–0.37]	68	S	0.05 [0.03–0.09]	20
Porcellio scaber	Isopoda (Porcellionidae)	F / I	0	0.84	2	0.2 [0.2-0.2]	2			0.03 [0.00-0.12]	59	S	0.07 [0.03–0.13]	10
Quedius brevis	Coleoptera (Staphylinidae)	O / I	2	0.93 [0.92-0.94]	4	11.2 [5.4-14.4]	3	0.93 [0.73–0.99]	14	0.00 [0.00–0.10]	35	H	0.82 [0.74–0.88]	12
Stenus aterrimus	Coleoptera (Staphylinidae)	O / I	4	0.88 [0.84-0.92]	7	0.4 [0.2-0.7]	7	0.00 [0.00-NA]	22	0.10 [0.03–0.22]	50	H	0.13 [0.08–0.18]	20
Thiasophila angulata	Coleoptera (Staphylinidae)	O / I	3	0.49 [0.47-0.52]	11	80.7 [52.7-108.8]	8	0.98 [0.90–1.00]	41	0.37 [0.27–0.48]	91	S	0.45 [0.40–0.50]	35
Thyreosthenius biovatus	Araneae (Linyphiidae)	O / I	3	0.82 [0.75-0.88]	9	3.5 [1.1-10.7]	9	0.38 [0.20–0.58]	21	0.22 [0.12–0.36]	54	H	0.24 [0.19–0.29]	26
Xantholinus linearis	Coleoptera (Staphylinidae)	F / I	0	0.33	1	79.5	1							

Biology: obligate myrmecophile (O), facultative myrmecophile (F), intranidal biology (I), extranidal biology (E). Bray-Curtis dissimilarity and cuticular hydrocarbon (CHC) concentration was determined in this study. Trophic role: S = scavenger, H = hunters: hunting on other myrmecophiles and scavenging. Host specificity was based on Parmentier et al. (2014): strict specialist = 4, specialist = 3, moderate specificity = 2, generalist =1, facultative myrmecophile = 0. Brood predation tendency, proportion individuals in brood chamber and proportion aggressive interactions were determined in Parmentier et al. (2016b), trophic role was based on Parmentier et al. (2016a). (*) the proportion of aggressive interactions towards larvae without protective case was extremely high: 0.87 [0.70–0.96], $N = 3$

total sample mass of compounds eluting between *n*-C20 and n-C40. The identification and quantification of CHCs with longer chains was impossible because of the limitations of the used column and our GC/MS-system.

This range was chosen based on the fact that there is direct behavioral evidence that CHCs in this range contain colony-specific and species-specific nestmate recognition cues in both mound-building *Formica* ants (Martin et al. 2008c) and in *Formica* ants living in soil nests (Akino et al. 2004b). An important role of these hydrocarbons in nestmate recognition of red wood ants and related mound-building *Formica* species has also been suggested in other studies (Martin et al. 2008a; Nielsen et al. 1999; Sorvari et al. 2008). Myrmecophiles that mimic their RWA host can, therefore, be expected to have a matching CHC profile in this range. One study reported that the cuticle of red wood ants also contain some compounds longer than *n*-C40 (Sutton et al. 2013), but it remains unknown, whether any of these have a clear biological function, and we were unable to quantify or identify these compounds due to the analytical limitations mentioned above.

Testing the Nature of the Observed Chemical Integration Strategies

A priori, we expected that chemical mimicry should translate into a high chemical similarity between hosts and parasites, whereas chemical insignificance should be reflected in low amounts of CHCs. To be able to test which of these chemical integration strategies applied to our study species, we quantified chemical dissimilarity for each myrmecophile individual based on the mean Bray-Curtis dissimilarity to the CHC profiles of host colony workers, using square-root transformed relative quantities (in ng). This transformation was used to down weigh the effect of very large peaks and which was selected due to the fact that it preserves quantitative information and can also deal with zero values. The amount of CHCs per unit of cuticular surface area ("CHC concentration", in ng/mm^2, i.e. corrected for the variation in body size) was calculated by dividing the estimated absolute CHC amounts (ng) by the individually estimated cuticular surface area or by the sum of estimated cuticular surface areas in pooled samples. Surface areas were determined using a Wild M3 binocular stereomicroscope with a measuring eyepiece and done by subdividing the bodies of the animals into basic geometric shapes, cf. detailed methodology and data given in Supplementary Material 2.

Chemical Strategy and Host Aggression

Here we tested the correlation of chemical dissimilarity (Bray-Curtis dissimilarity) and concentration of CHCs (ng/mm^2 cuticle) with host aggression in order to find general patterns in the chemical integration mechanisms used by the different species studied. The dependent variable "Host aggression" was already determined in Parmentier et al. (2016b). Ant aggression was scored by the proportion of aggressive host ant interactions (acid spraying, chasing, biting, opening mandibles) out of the first 20 interactions towards a myrmecophile in controlled aggression trials. In addition to chemical dissimilarity and concentration of CHCs, we also included other functional traits as independent variables in our model. These functional traits included taxonomic group (family level), host specificity, brood predation tendency, the level of nest integration, trophic role and body size. Host specificity was based on a previous literature study which summarized all known ant hosts of myrmecophiles associated with RWAs (Parmentier et al. 2014). For this study, we categorized the tested myrmecophiles in 4 categories with different degrees of host specificity and assigned a rank: strict specialist = 4: only records with RWAs; specialist = 3: some records with non RWAs, but RWAs are the main host; moderate specificity = 2: records with RWAs, but distribution in non-RWAs probably important as well; generalist = 1: myrmecophiles with a broad host spectrum. Brood predation tendency of a myrmecophile species was quantified as the proportion of individuals that preyed on eggs of red wood ant (Parmentier et al. 2016b). The level of nest integration reports the proportion of individuals that preferred densely populated chambers with ant brood to less crowded chambers without brood (Parmentier et al. 2016b). The trophic role was determined by offering different food sources (Parmentier et al. 2016a). Species that fed on ant brood and dead prey were categorized as scavengers. Species that preyed on other myrmecophiles (and also scavenged) were classified as hunters. Body size (mm^2) was estimated as the total cuticular surface (see above and Supplementary Material 2).

Statistical Analyses

To visualise the chemical similarities of the complete CHC profiles, a hierarchical cluster analysis was calculated from the Bray-Curtis dissimilarity matrix of the standardized CHC quantities using the unweighted pair group method (UPGMA) algorithm and the *hclust* function in R. Apart from the similarity of the total set of hydrocarbons, we examined the pattern of similarity within a subset of different classes of hydrocarbons with separate cluster analyses. These classes included *n*-alkanes, methyl-branched alkanes, dimethyl-branched alkanes and *n*-alkenes. This was done to allow for the fact that ants might use only a subset of the hydrocarbons to recognize nestmates (Martin et al. 2008b; Guerrieri et al. 2009) and that myrmecophiles could

achieve chemical mimicry merely by matching a part of the total bouquet. Peaks of a particular subset were square root transformed and divided by the total (square root transformed) amount of compound present belonging to that class in the profile. For each myrmecophile species, the significance of the CHC similarity with the host ant workers was determined using a *PERMANOVA* with the *adonis* function in R package vegan 2.3–2, using the Bray-Curtis dissimilarities between the standardized CHC abundances, and using a maximum of 9999 permutations (less only if there were too few samples to carry out this number of permutations, Table 3). Most myrmecophiles were collected from nests of two or even three RWA host species. To account for possible species-specific chemical adaptations to their RWA host ant species, we incorporated a factor "host species" in the strata argument of the *adonis* function. This factor grouped RWA workers and myrmecophile individuals in three levels, i.e. samples collected in nests of *F. rufa*, *F. polyctena* and *F. pratensis*. The strata argument only allowed permutations among samples (myrmecophile and workers) within the levels of this grouping factor. A more detailed grouping of workers and myrmecophiles per nest dramatically reduced the maximum number of permutations which could be carried out per species. Hence, we tested the differences between RWA workers and myrmecophiles across nests of the same RWA host species rather than across individual nests. The sample sizes of these tests are listed in Table 3.

The histerid beetles *Myrmetes paykulli* and *Dendrophilus pygmaeus* (Histeridae) had many compounds in common with their hosts, but some compounds were present in trace quantities, i.e. lower than the 0.1% sample mass threshold or were lacking. In addition, some compounds were lacking or present in trace amounts in some ant samples and were not included in the original dataset. To avoid that the absence of compounds or trace compounds could affect the analysis of these chemically matching species, we focused in a more detailed analysis on the CHCs that were present in all RWA worker samples and histerid beetle samples. To have a maximum number of CHCs shared by all RWA samples and histerid beetle samples, we also included hydrocarbons present in a quantity lower than the 0.1% of the sample mass. We ran similar cluster analyses on the Bray-Curtis dissimilarity matrix of this CHC dataset shared by the three RWA species, *M. paykulli,* and *D. pygmaeus* for the complete CHC profile and different subsets of the CHC profile (*n*-alkanes, methyl-branched alkanes, dimethyl-branched alkanes and *n*-alkenes). The Bray-Curtis dissimilarity matrices were again based on square root transformed data divided by the total sample amount of CHCs, shared by histerid beetles and RWAs or the total amount of a subset of shared CHCs for the subset analyses. Because of the high similarity in the profile of RWA workers and beetles, these myrmecophiles might rely not only on species-specific but also on colony-specific adaptations to the chemical profile of the supporting colony. As a result, differences between workers and either *M. paykulli* or *D. pygmaeus* were tested with a *PERMANOVA* in which we included the factor "host colony" in the strata argument of the *adonis* function.

For each myrmecophile species, we also compared its CHC concentrations (ng/mm^2) with the CHC concentrations (ng/mm^2) of RWAs using a *Wilcoxon rank sum test* with continuity correction.

Finally, the effect of different functional traits, including Bray-Curtis dissimilarity and CHC concentration, on host aggression was assessed with a quasibinomial linear model with logit link function using function glm. Only main effects were considered in the model. The most parsimonious model was selected with an exhaustive search based on the quasi Akaike Information Criterion (qAIC), using R package glmulti. Significance was assessed using Type II likelihood ratio tests with the Anova function in R package car. Extranidal myrmecophiles and *Emphylus glaber* were not included in this analysis as data for the controlling variables were missing (Table 2). The larva of the leaf beetle *C. quadripunctata* was also not considered as it lives enclosed in a case made of ant nest material and ants do not directly detect the chemical composition of the larvae. The variable "CHC concentration" was transformed by dividing the concentrations by the CHC concentration of RWAs and by subsequently log 10 transforming these relative CHC concentrations. Body size was also log10 transformed. Confidence intervals of the BC dissimilarity to the host workers and the CHC concentrations reported in Table 2 were estimated via bootstrapping using package boot. Confidence intervals of the other parameters of Table 2 were taken from earlier studies.

All statistical analyses were done in R version 3.2.1 (R Core Team 2014). *P*-values for analyses where we used multiple repeated tests, i.e. for our *PERMANOVA tests* and *Wilcoxon rank sum tests*, were corrected for multiple testing using the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995).

Results

Characteristics of the Chemical Profile

In total, 118 different GC-peaks, representing CHCs were attributed across all our samples. Supplementary Material 1 provides an overview of hydrocarbons, whereas Supplementary Material 3 shows the percent composition for each hydrocarbon. Some peaks contained several CHCs that could not be separated under the described GC/MS-

Table 3 Results of *Permanova* Tests which Compared Pairwise the Differences in Cuticular Hydrocarbon Composition Between Ants and Myrmecophile Species

Species	Permutations	*N*	Host species			P
			F. polyctena	*F. pratensis*	*F. rufa*	
Red wood ant workers		46	26	7	13	
Amidobia talpa	9999	4	1		3	<0.001
Clytra quadripunctata adult	8568	5			5	<0.001
Clytra quadripunctata larva	378	2			2	0.004
Coccinella magnifica adult	9999	14	8	6		<0.001
Coccinella magnifica larva	9999	8	8			<0.001
Cyphoderus albinus	5292	3	2		1	<0.001
Dendrophilus pygmaeus	378	2	2			0.027
Dinarda maerkelii	9999	6	3		3	<0.001
Emphylus glaber	560	3				0.003
Leptacinus formicetorum	378	2	1		1	<0.001
Lyprocorrhe anceps	9999	4	1	1	2	<0.001
Mastigusa arietina	9999	5	5			<0.001
Monotoma angusticollis	9999	8	6	1	1	<0.001
Monotoma conicicollis	378	2	2			0.003
Myrmetes paykulli	9999	9	5	2	2	0.002
Notothecta flavipes	9999	7	4		3	<0.001
Pella humeralis	27	1	1			0.039
Platyarthrus hoffmannseggii	9999	8	5	1	2	<0.001
Porcellio scaber	378	2	2			0.004
Quedius brevis	9999	4	1		3	<0.001
Stenus aterrimus	9999	7	2	2	3	<0.001
Thiasophila angulata	9999	11	4	1	6	<0.001
Thyreosthenius biovatus	9999	9	5	1	3	<0.001
Xantholinus linearis	14	1			1	0.071

Number of unique permutations, total number of myrmecophile samples (N) and number of samples associated with each red wood ant host are given. Permutations among workers and samples of a myrmecophile species were only allowed within the same level of host species. Benjamini-Hochberg corrected P-values are given in the last column (P)

conditions. Red wood ants (RWAs) possessed most CHC peaks (*F. rufa* = 86, *F. polyctena* = 87, *F. pratensis* = 82) together with the histerid beetles *M. paykulli* (*N* = 87) and *D. pygmaeus* (*N* = 78) (Supplementary Material 1 and 3). *M. paykulli* had 83 out of 87 compounds in common with RWAs and *D. pygmaeus* 76 out of 78. As expected, the profiles of RWA workers comprised almost uniquely CHCs (e.g. *F. polyctena* 0.97, CI: 0.97–0.98). Myrmecophiles, however, varied vastly in the proportion of hydrocarbons. That is, while profiles of some of the species consisted mainly of CHCs, akin to the situation for the ant hosts (e.g. proportion of CHC compounds in *M. paykulli*: 0.95, CI: 0.93–0.96), for other species non-CHCs almost completely dominated the profile (e.g. proportion of CHC compounds in the rove beetle *Quedius brevis*: 0.03, CI: 0.00–0.04). The exact nature of these compounds would require further studies, as we did not have sufficient material available to unambiguously identify these compounds. In addition, we cannot exclude that some of these compounds originated from glands rather than from the insect cuticle. Characteristic gas chromatograms of the RWA hosts and associated myrmecophiles are shown in Supplementary Material 1.

Testing the Nature of the Observed Chemical Integration Strategies

The hierarchical cluster analysis of the standardized CHC quantities separated the RWA workers clearly from most myrmecophiles (Fig. 1). Most RWA workers aggregated with workers of the same nest. Sample sizes of most myrmecophiles were relatively small, but differences in CHC compositions were generally very large and consistent across samples of the same myrmecophile species. The clear distinction in profiles between myrmecophiles and their host is confirmed by *PERMANOVA* tests (Table 2),

which showed highly significant differences in all myrmecophiles except for *Pella humeralis* ($P = 0.07$), where we had low statistical power due to the fact that only 15 unique permutations were possible for this species. Only the histerid beetles *M. paykulli* and *D. pygmaeus* aggregated within the RWA clusters (bold leaves in Fig. 1) and showed high similarity in their chemical profiles with RWAs (Supplementary Material 1 and 3). A similarly high dissimilarity between RWA workers and myrmecophiles was observed in the analyses only focusing on methyl-branched alkanes (40 peaks) and dimethyl-branched alkanes (28 peaks) (Supplementary Material Fig. S3-S4). RWA workers also grouped more or less with workers of the same nest in these analyses, and only *M. paykulli*, *D. pygmaeus* and some individuals of *Cyphoderus albinus* and *Platyarthrus hoffmannseggii* were found in the cluster grouping all RWAs. More myrmecophiles clustered with the red wood ants in the analyses limited to *n*-alkanes (17 peaks) and *n*-alkenes (20 peaks) of the CHC profile (Supplementary Material 4, Fig. S1-S2). These two analyses also gave a poorer distinction between worker profiles of different RWA nests, suggesting that *n*-alkanes and *n*--alkenes have a less promintent role in nestmate discrimination. More detailed cluster analyses focusing on the CHCs that RWA workers and the two histerid beetles had in common (55 peaks) were also performed. The RWA workers tend to cluster in distinct nest-specific profiles. *D. pygmaeus* and *M. paykulli* were not found within the cluster of the host nest, although the latter tend to group closer to their host nest than to other RWA nests (Fig. 2). Similar patterns were found for all subsets of the CHC profile (Supplementary Material 4, Fig. S5-S8). Permutation tests for all shared CHCs (55 peaks), shared *n*-alkanes (11 peaks), shared methyl-branched-alkanes (21 peaks), shared dimethyl-branched alkanes (14 peaks), and shared *n*-alkenes (5 peaks) showed that *M. paykulli* ($P < 0.005$ in all five tests, permutations = 9999) and *D. pygmaeus* ($P = 0.067$ in all five tests, lowest value possible as the max. Number of unique permutations was 15) were chemically different from host nest workers. In spite of their similarity in CHCs, they also elicited a significant aggression response (Table 2).

The estimated CHC concentration per mm^2 body surface varied greatly among all tested arthropods. RWAs were characterized, except for adults of *Clytra quadripunctata*, by the highest CHC concentration per mm^2 body surface (mean concentration ± SE: 228.6 ng/mm^2 ± 25.7, Table 2). Seventeen out of 21 myrmecophiles (for the ladybird *Coccinella magnifica* and the leaf beetle *C. quadripunctata* only the larvae had lower concentrations) had significantly lower CHC concentration than RWA workers (Supplementary Material 5). Some of this observed interspecific variation in CHC concentrations could result from the rather crude body surface estimates. The latter, however, could not explain the huge differences observed in ants and some myrmecophiles. Indeed, 10 obligate myrmecophiles (Table 2) had concentrations 10 to 1000-fold lower than that of RWAs. The lowest concentrations were found in the facultative isopod *Porcellio scaber* (mean concentration ± SE: 0.19 ng/mm^2 ± 0.02, Table 2). Some species (*P. scaber*, the rove beetle *Stenus aterrimus,* the root-feeding beetles *Monotoma angusticollis* and *M. conicicollis*, the isopod *P. hoffmannseggii*, the spider *Thyreosthenius biovatus* and the springtail *C. albinus*) with very low concentrations of hydrocarbons per mm^2 of cuticle were mostly ignored in aggression trials. However, other species with very low hydrocarbon and non-hydrocarbon concentrations were immediately detected and persecuted (e.g. the spider *M. arietina* and the beetle *Q. brevis*). Non-hydrocarbons could have different effects on ants ranging from elevated aggression to repelling or to appeasing (Akino 2008; Stoeffler et al. 2011).

Chemical Strategy and Host Aggression

Our most parsimonious model contained the factors log10 (relative CHC concentration), trophic role, Bray-Curtis dissimilarity and proportion of brood predation. Both log10 (relative CHC concentration) and trophic role contributed significantly to host aggression. Lower CHC concentrations (LR $\chi^2 = 9.00$, $P = 0.003$) and hunters (LR $\chi^2 = 6.48$, $P = 0.011$) provoked less aggression (Fig. 3)a,b. Aggression increased with increasing Bray-Curtis dissimilarity and higher proportion of brood predation, but neither relationship quite reached statistical significance (LR $\chi^2 = 3.30$, $P = 0.07$; LR $\chi^2 = 3.10$, $P = 0.08$) (Fig. 3)c,d. The spider *Mastigusa arietina* was an outlier in Fig. 3a, c and d.

Discussion

The present study sheds light on the chemical integration strategies used by arthropods associated with red wood ants (RWAs). Previously, most arthropods associated with social insects have been found to make use of chemical mimicry to integrate into the nests of their host (claimed for 50 out of 61 species tested, see Table 1). By contrast, results of the present study, which focused on arthropods that had a relatively loose association with RWAs, showed that only two of our study species displayed CHC profiles that were similar to those of their host and that the majority did not match the host's chemical profile at all.

The CHC profiles of the three tested RWA species was highly congruent with those reported in earlier studies (Martin et al. 2008b; Włodarczyk 2011). RWA workers

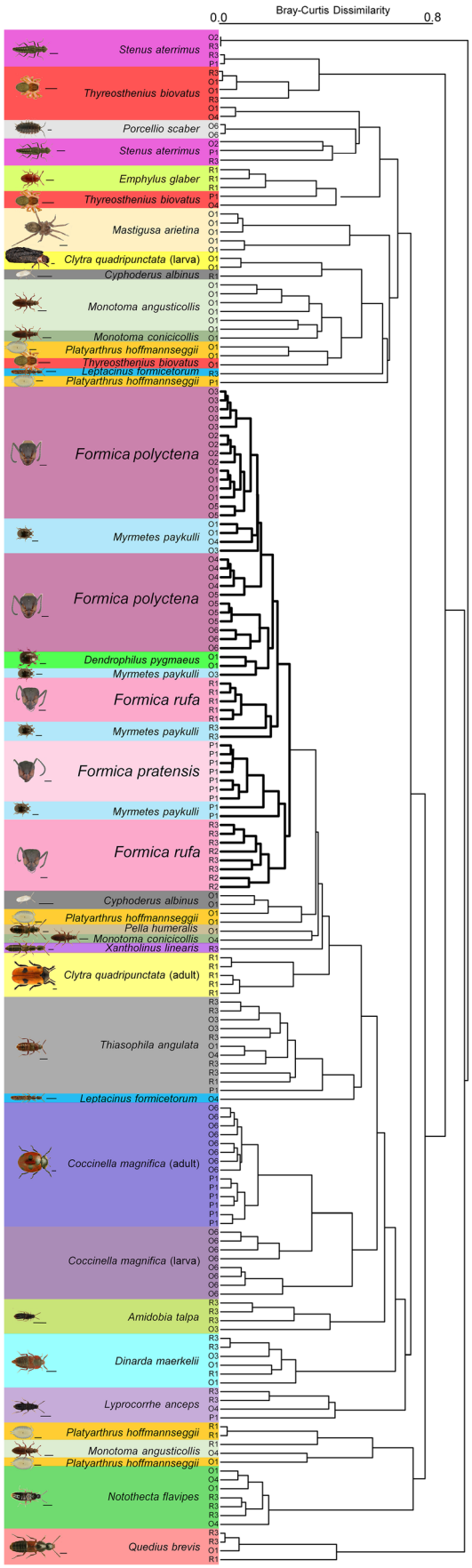

Fig. 1 Hierarchical cluster analysis of the cuticular hydrocarbon profiles of red wood ant host species and associated intranidal (inquilines) and extranidal guests. Clustering was conducted with the unweighted pair group method with arithmetic mean and the Bray-Curtis dissimilarity matrix. Scale bars under figures represents a length of 1 mm. Letter code refers to the nest where the sample was collected (O1–6: *Formica polyctena* nests, P1: *Formica pratensis* nests, R1–3: *Formica rufa* nests). Cluster with the thicker leaves groups all red wood ants. Ant figures were adapted from www.AntWeb.org, myrmecophile figures were adapted from pictures kindly provided by Lech Borowiec or by the first author

of the same colony also clustered strongly, based on their similarity in CHC profiles, in line with their pivotal role in nestmate recognition (Martin et al. 2008a). Martin et al. (2008b) reported that chemical species identity in RWAs is mainly based on dimethyl-alkanes. We found that particularly the dimethyl-branched and methyl-branched alkane fraction of the CHC profile were more or less colony-specific. The histerid beetles *D. pygmaeus* and *Myrmetes paykulli* had almost all components in common with the RWA workers. However, the chemical profiles were distinct from those of their host colony. Chemical mimicry is defined as adaptive resemblance of the CHC profile of a myrmecophile to their host (von Beeren et al. 2012b). But it is not clear whether the two histerid beetles benefit from the resemblance in CHCs. The beetles were regularly detected and even bitten by the ants (pers. observations TP). Moreover, *F. rufa* aggression towards *M. paykulli* individuals found in the same nest was not lower than towards individuals transferred from a *F. polyctena* colony (Parmentier et al. 2016b). The chemical profile of the 18 other obligate RWA myrmecophile species was clearly different from their RWA host in terms of CHC composition (Supplementary Material 4, Fig. 1). In contrast to RWAs, non-hydrocarbons also contributed significantly to the overall profile of these myrmecophiles. Interestingly, none of these 18 species closely matched the CHC profiles of their RWA hosts (Fig. 1), which was already indicated by aggression transfer experiments between different red wood ant hosts previously performed in 11 species of this group (Parmentier et al. 2016b). However, some myrmecophiles only partially mimic their host (Pérez-Lachaud et al. 2015). The positive trend between increasing chemical distance and host aggression in Fig. 2C suggest that some mimicry might be better than none. The majority of the myrmecophiles were characterized by significantly lower estimates of CHC concentrations than their host. We found that lower concentrations of CHCs were correlated with lower host aggression and therefore, some species may associate with their host via a strategy of chemical insignificance. Ants may discriminate very low CHC concentrations, as shown in *Aphaenogaster senilis* which detected alien CHC

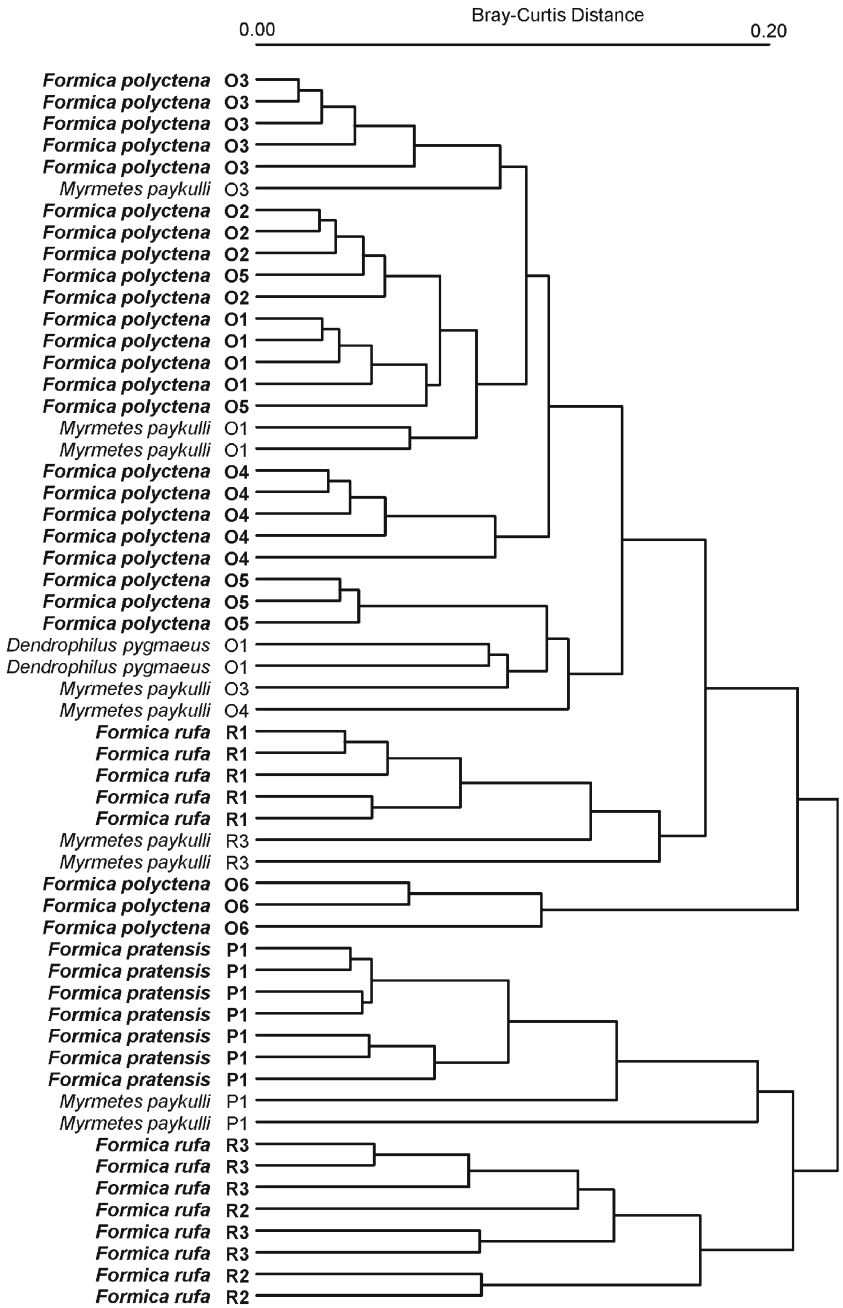

Fig. 2 Hierarchical cluster analysis of the cuticular hydrocarbons that are shared by three red wood ant host species and two histerid beetles. Clustering was conducted with the unweighted pair group method with arithmetic mean and the Bray-Curtis dissimilarity matrix. Letter code refers to the nest where the sample was collected (O1–6: *Formica polyctena* nests, P1: *Formica pratensis* nest, R1–3: *Formica rufa* nests)

concentrations at a concentration of 0.05 ng/mm^2 (Ichinose and Lenoir 2010). This concentration is tenfold lower than the lowest CHC concentrations measured in the tested myrmecophile community. The effectivity of having low CHC concentrations as an integration strategy is, therefore, uncertain. But the same study indicated that aggression significantly dropped at lower CHC concentrations, which suggests that low CHC concentrations might indeed be beneficial. These low CHC concentrations might have an adverse effect. It could affect the sensitivity to desiccation, which is strongly controlled by the concentration of hydrocarbons (Blomquist and Bagnères 2010). Indeed, we observed that some myrmecophiles, such as *P. hoffmannseggii* and *C. albinus* were very sensitive to drought in the lab. Surprisingly, some species such as the spider *M. arietina* and the beetle *Q. brevis* were heavily aggressed, bitten, and even chased in spite of low CHC-concentrations. High aggression towards these species could be caused by the emission of volatiles, non-detected compounds or by non-hydrocarbon compounds that could elicit strong aggression even in low concentrations. In addition, aggression could be affected by the life style of these myrmecophiles, as we found that species that hunt on other myrmecophiles were more heavily

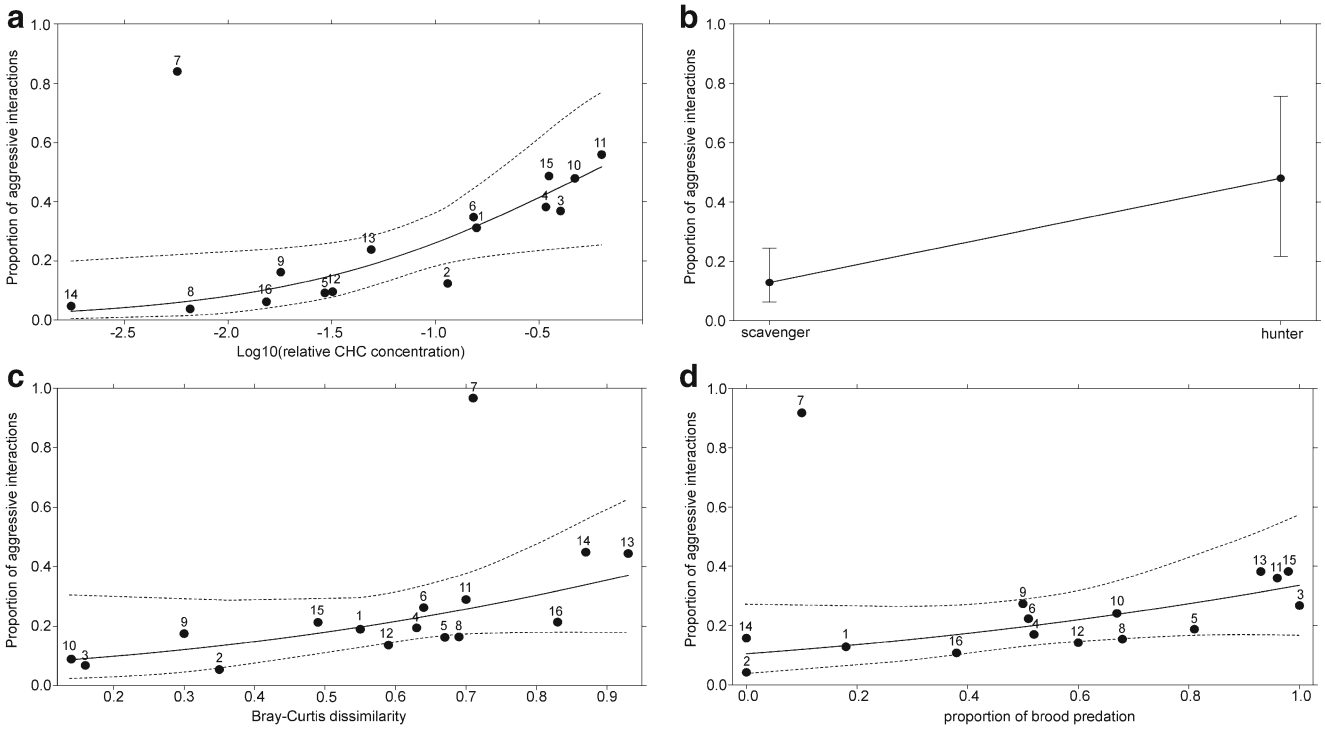

Fig. 3 Residual plots showing the association between host aggression and the four factors retained in the most parsimonious model. Dashed lines indicate upper and lower 95% confidence limits. a) correlation between log10(relative CHC concentration) and host aggression (P = 0.003). b) correlation between trophic role and host aggression (P = 0.011). c) correlations between Bray-Curtis dissimilarity and host aggression (P = 0.07). d) correlation between proportion of brood predation and host aggression (P = 0.08). Myrmecophile's label: 1. *Amidobia talpa* 2. *Cyphoderus albinus* 3. *Dendrophilus pygmaeus* 4. *Dinarda maerkelii* 5. *Leptacinus formicetorum* 6. *Lyprocorrhe anceps* 7. *Mastigusa arietina* 8. *Monotoma angusticollis* 9. *Monotoma conicicollis* 10. *Myrmetes paykulli* 11. *Notothecta flavipes* 12. *Platyarthrus hoffmannseggii* 13. *Quedius brevis* 14. *Stenus aterrimus* 15. *Thiasophila angulata* 16. *Thyreosthenius biovatus*

attacked than scavengers. In general, hunters are more active and move faster, which might facilitate their detection. Naked larvae of the leaf beetle *Clytra quadripunctata* were fiercely attacked, but are normally protected by a case made of excrement and nest material (Donisthorpe 1927) that does not attract the attention of ant workers (Table 1). Rather than matching the profile of the worker caste, myrmecophiles might in principle also target the sexual castes (Hojo et al. 2009) or ant brood (Nash et al. 2008). However, the CHC composition of brood and sexuals only shows minor differences in most social insect species (Elmes et al. 2002; Hojo et al. 2009; Van Oystaeyen et al. 2014) and could not explain the vast differences we observed in the majority of the myrmecophiles. Alternatively, the chemical profile of myrmecophiles might resemble the odor of the nest material. This strategy seems to be employed by an extranidal caterpillar which mimics the odor of its host plant and in this way evades detection by predatory ants (Akino et al. 2004a). Though we cannot rule out such mechanism in our community, we consider this hypothesis unlikely, given that nest material in ant nests gets coated with host-specific cuticular hydrocarbons (Lenoir et al. 2009; Bos et al. 2011). Moreover, the aggression response of ants towards the myrmecophiles was similar in the presence of nest material (pers. observations TP). In contrast to many parasites that want to mask their identity in the nest (cf. Table 1), mutualists can produce distinct compounds or profiles to attract their partner ant species (Hojo et al. 2014; Richard et al. 2007). Some of the RWA myrmecophiles might provide some indirect mutualistic services (Parmentier et al. 2016a) or they might even mimic the distinct profile of true mutualists to mask their identity as was shown in aphid predators (Liepert and Dettner 1996; Lohman et al. 2006). However, none of the RWA myrmecophiles were treated (grooming, transporting, antennae tapping) as mutualists by the ants (Parmentier et al. 2016b). Therefore, we argue that the tested myrmecophiles do not carry or imitate a distinct "mutualist" chemical profile. Finally, we did not find evidence that RWA myrmecophiles only match a particular structure class of CHCs.

It is surprising that in our study system only 2 out of 18 inquiline arthropods closely matched the CHC profiles of their host, even though this strategy had been reported for most arthropods living in social insect nests studied up till now. This discrepancy could be explained by the specific structure of a RWA nest of which the aboveground

part is a dome-shaped mound constructed with organic material, needles, twigs, and other plant material (Gösswald 1989). This haystack-like structure provides many more hiding places for myrmecophiles than typical soil nests (Seifert 2007). Detection of myrmecophiles could further be hampered by the relatively large size of RWAs (Parmentier et al. 2016c). Hence, RWA myrmecophiles might not require chemical mimicry as they could easily run away or hide when detected. However, the underground part of a RWA nest is very similar to a classic underground ant nest, and most of the inquilines used in this study were also found there. Moreover, the majority of RWA inquilines can easily live in chambers with high densities of workers (Parmentier et al. 2016b). Hence, we believe that the aforementioned discrepancy can mainly be explained by a biased focus in the literature on chemical strategies of fairly specialized arthropod inquilines (Table 1). Indeed, the intense interaction of these species with their host is likely only possible by chemically matching the host, whether or not combined with advanced adaptations at the behavioral or morphological level. Although unspecialized species might outnumber the group of specialized associates (; (Wasmann 1894; Kistner 1979; Parmentier et al. 2014), little is known about the chemical integration strategies they employ. The CHC profile of three myrmecophilous beetles that live in the vicinity of the nests of *Lasius fuliginosus* showed no apparent similarity in CHC composition with their host (Stoeffler et al. 2011). The authors suggested that these extranidal beetles show no disguise as they have plenty of hiding places outside the nest and hardly interact with their host compared to inquilines found inside nests. Our results indicate that unspecialized associates can also survive as inquilines inside densely populated nests without mimicking the CHC profile, and some of them even prefer the deepest parts of the nest (Table 2). Some might be chemically insignificant but others showed no cuticular chemical disguise. These species might have a similar profile compared to free-living relatives. This is suggested by the slight difference in CHC that we observed between the obligate myrmecophilous ladybird *C. magnifica* and its free-living sister species *C. septempunctata* (Supplementary Material 1 and 3).

Overall, our study suggests that the transition towards a myrmecophilous life history does not require the matching of the host recognition cues. Further studies should compare in-depth the chemical and behavioral strategies of unspecialized myrmecophiles with their free-living relatives. It is likely that unspecialized myrmecophiles might rely on traits or tactics already present in their free-living relatives such as low concentrations of CHCs, tergal glands or specific defensive behavior. These tactics might be sufficient to penetrate and exploit a colony and might be the onset of the evolution towards advanced chemical (special glands, chemical mimicry), morphological, and behavioral strategies needed for a complete assimilation into colony life as seen in most specialized myrmecophiles (Parker 2016).

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